IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	: Yoshiyuki Nagai et al.	Art Unit	: 1632
Serial No	: 09/132,521	Examiner	: Joseph Woitach
Filed	: August 11, 1998		
Title	: RECOMBINANT SENDAI VIRUS VECTOR EXPRESSING CHEMOKINE		

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132

1. I am a research scientist at DNAVEC Research Inc., the assignee of the above-referenced application. I have technical experience in the research fields of Molecular Biology, Virology, Biochemistry, Protein Chemistry, etc. I am also an author of over nine publications, with over two years experience in research concerning the Sendai virus vector. A copy of my curriculum vitae is attached as Appendix A.

2. I am making this Declaration to provide relevant facts in support of the patentability of the subject matter claimed in the patent application.

3. I have read and understood the outstanding Office Action mailed on June 5, 2001.

4. I understand that the Examiner contends the inventions of claims 1-8, 11, 12, 14, and 15 to be unpatentable over Yu et al. in view of Bluel et al.

5. To overcome this obviousness rejection and to demonstrate that generally, protein expression by the Sendai virus cannot be foreseen, I submit the experiment data described below.

6. In this experiment, the production efficiency of nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) when using a recombinant Sendai virus vector was determined as follows.

7. Sendai virus vectors containing a mouse nerve growth factor (NGF) gene (SeV/NGF) or a mouse glial cell line-derived neurotrophic factor (GDNF) gene (SeV/GDNF) were prepared as follows. A NotI fragment comprising the NGF gene or the GDNF gene was amplified by PCR with the following primers containing Sendai virus-specific transcriptional regulation signal sequences.

Mouse NGF
N-terminal primer:
5'- ACTTGCGGCCGCCAAAGTTCAGTAATGTCCATGTTGTTCTACACTCTG –3'
C-terminal primer:
5'- ATCCGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTCAGCCTCT
TCTTGTAGCCTTCCTGC –3'

Mouse GDNF
N-terminal primer:
5'- AGTTGCGGCCGCCAAAGTTCAATGAAGTTATGGGATGTCGTGG –3'
C-terminal primer:
5'- ACGTGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGGGGTCAGAT

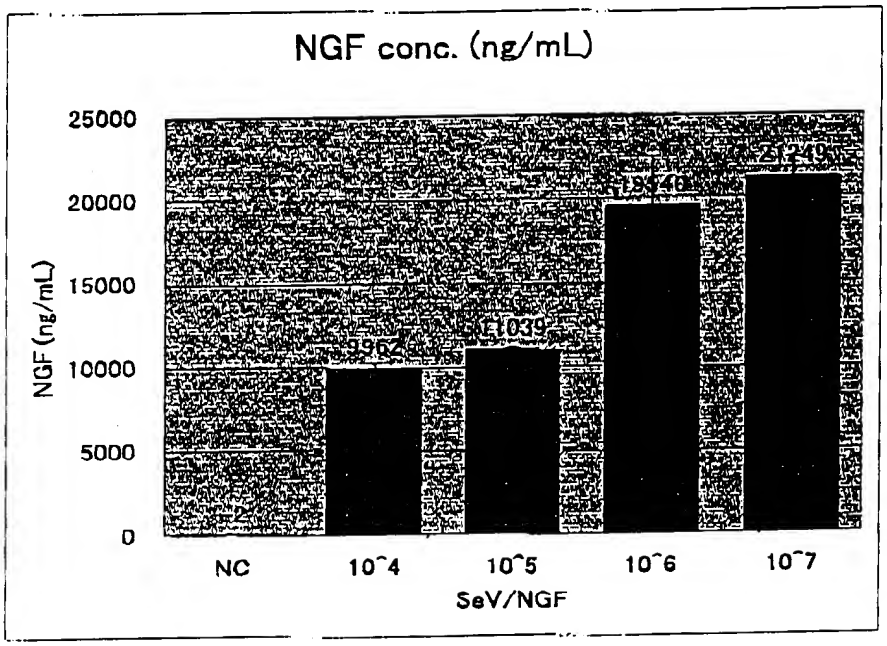
ACATCCACACCGTTTAGCGG -3'

The NotI fragment was cloned in the NotI site of pSeV18+b(+), which has been constructed to produce the exact Sendai virus full-length antigenomic plus sense RNA of 15402 nucleotides (Hasan, M.K. et al., J. Gen. Virol. 78: 2813-2820 (1997)). The resulting plasmid pSeV/NGF or pSeV/GDNF was transfected into LLC-MK2 cells previously infected with vaccinia virus vTF7-3, expressing T7 polymerase. The T7-driven full-length recombinant SeV/NGF or SeV/GDNF RNA genomes were encapsulated with NP, P and L proteins which were driven from the respective cotransfected plasmids. Seventy-two hours later, the transfected cells were injected into embryonated chicken eggs, resulting in the recovery of the virus comprising the desired gene (Kato, A. et al., Genes Cells 1: 569-579 (1996)).

8. The Sendai virus-mediated gene expression of NGF and GDNF was measured as follows. SeV/NGF or SeV/GDNF was injected into embryonated chicken eggs (E7) at $1x10^4$, $1x10^5$, $1x10^6$ and $1x10^7$ CIU/egg, where CIU means cell infectious units. Seventy-two hours later, the allantoic fluid of the eggs was collected. To measure the protein concentrations of NGF or GDNF in the allantoic fluid, a standard ELISA assay was performed basically according to the manufacture's recommendations (Promega, WI, U.S.A.).

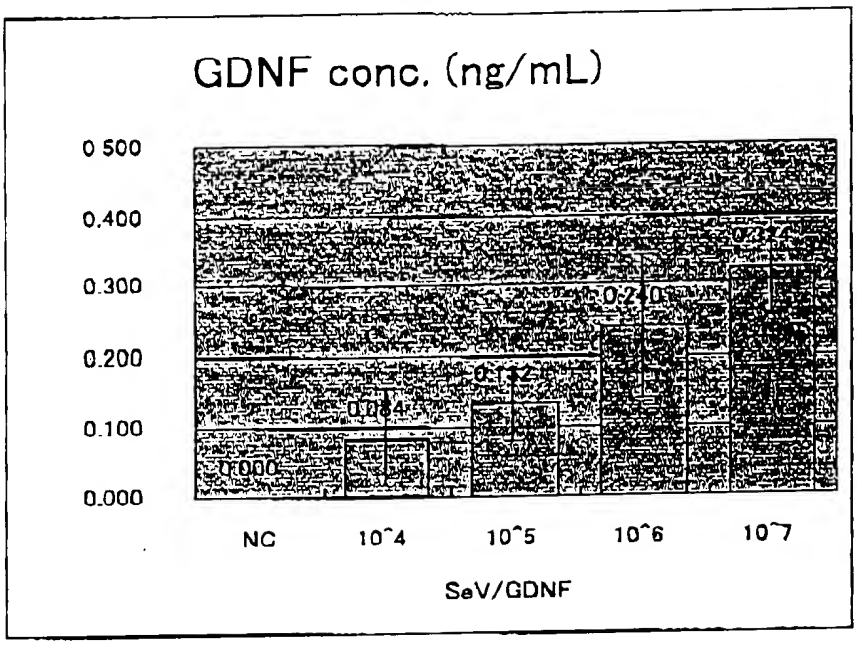
9. Figure 1 shows the production efficiency of NGF 72 hrs after infection.

Fig. 1



10. Figure 2 shows the production efficiency of GDNF 72 hrs after infection.

Fig. 2



11. The above data are summarized in Table 1 below.

Table 1

Infected viral conc. (pfu/ml)	NC	10^4	10^5	10^6	10^7
GDNF conc. (ng/ml)	0.00	0.084	0.132	0.240	0.324
NGF conc. (ng/ml)	-2	9962	11039	19540	21249

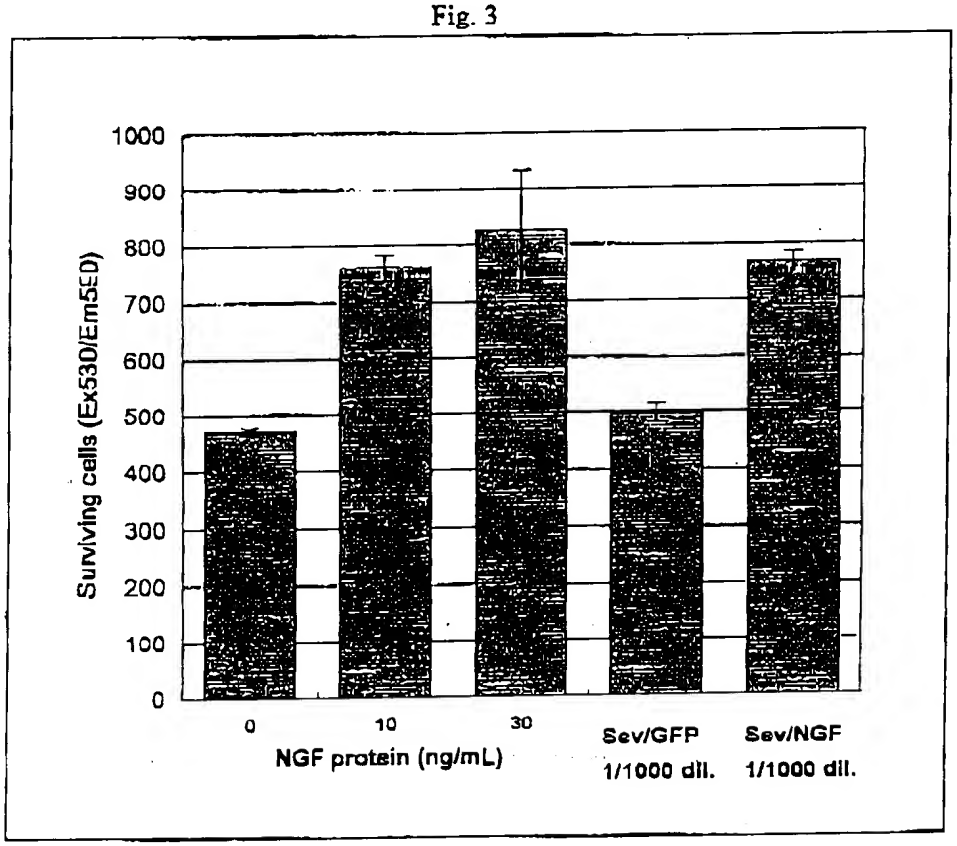
NGF was produced 60,000 to 80,000 times better than GDNF. There was no difference in the proliferation of recombinant viruses after infection. Hemagglutination test (HA), a viral assay, revealed an equivalent titer (2^{11} dilution), indicating that a foreign gene does not affect the replication of the Sendai virus. Thus, the above data show that although the exogenous gene has no influence on the replication of the Sendai virus itself, it influences the production efficiency of the proteins when using a Sendai vector, to an extent that the effect of the vector-protein combination cannot be predicted beforehand.

12. The Sendai virus-mediated gene expression of NGF was further measured as follows. LLC-MK2 cells infected with SeV/NGF at m.o.i 0.01 were cultured for 3 days with serum-free MEM containing 7.5 μg/ml trypsin (GIBCO). After the 3-day culture, in which almost 100% of cells were infected, medium was changed to serum-free MEM medium without trypsin and further cultured for 3 days. Then, the culture supernatant was recovered and centrifuged at 48,000x g for 60 min. The protein concentration of NGF in the supernatant was measured by the standard ELISA assay as descried above. As a result, 10.5 μg/ml of NGF protein was detected in the culture supernatant. This indicates that the amount of protein produced by the SeV/NGF is considered to be a "substantial amount."

13. It was shown as follows that the NGF produced with Sendai virus vectors was biologically

active. The measurement of *in vitro* activity of NGF protein was accomplished by using a dissociated culture of primary chicken dorsal root ganglion (DRG; a sensory neuron of chicken) using survival activity as an index (Nerve Growth Factors (Wiley, New York), pp 95-109 (1989)). Dorsal root ganglion was removed from day 10 chicken embryo, and dispersed after 0.25% trypsin (GIBCO) treatment at 37°C for 20 min. Using high-glucose D-MEM medium containing 100 units/ml penicillin (GIBCO), 100 units/ml streptomycin (GIBCO), 250 ng/ml amphotericin B (GIBCO), 20 mM 2-deoxyuridine (Nacalai Tesque), 20 mM 5-fluorodeoxyuridine (Nacalai Tesque), 2 mM L-glutamine (Sigma), and 5% serum, cells were seeded onto 96-well plate at about 5000 cells/well. Polylysin-precoated 96-well plates (Iwaki) were further coated with laminin (Sigma) before use. At the start point, control NGF protein or previously prepared culture supernatant after Sendai virus infection was added. After 3 days, cells were observed under a microscope, and quantification of surviving cells was conducted by adding Alamar Blue (CosmoBio) using the reduction activity by mitochondria as an index (measuring 590 nm fluorescence, with 530 nm excitation). Equivalent fluorescence signals were obtained in control (without NGF addition) and where 1/1000 diluted culture supernatant of cells infected with Sendai virus vectors containing a green fluorescent protein (GFP) gene (SeV/GFP) was added, whereas the addition of 1/1000 diluted culture supernatant of cells infected with SeV/NGF caused a notable increase in fluorescence intensity, and it was judged that a high number of cells had survival activity (Figure 3). The value of effect was comparable to the value when an equal amount of NGF protein to that calculated from ELISA was added.

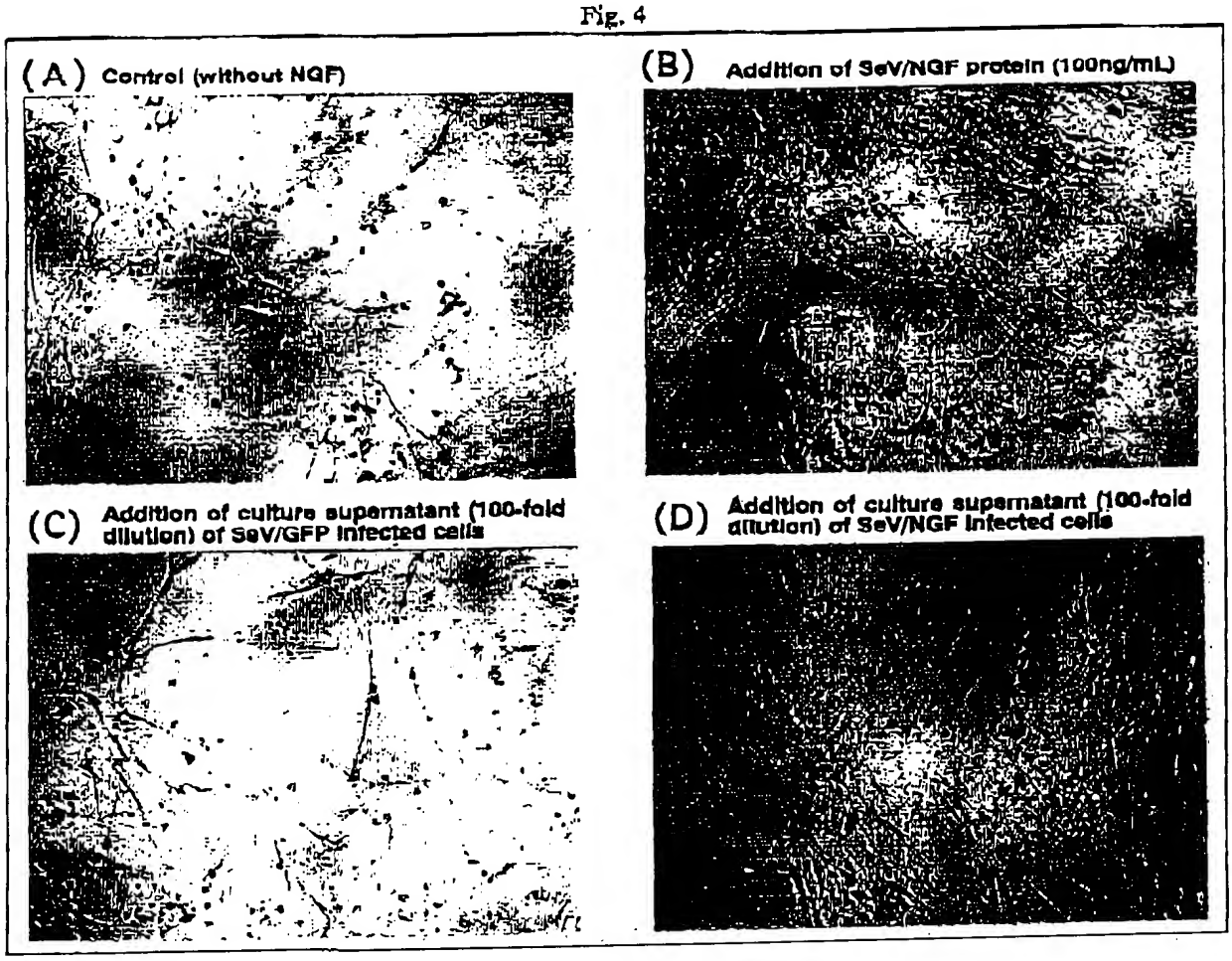
Fig. 3



Observation under a microscope proved a similar effect. Namely, by adding culture

supernatant of cells infected with SeV/NGF, increase in surviving cells and notable neurite elongation were observed (Figure 4).

Fig. 4



Thus, it was confirmed that NGF expressed after infection of NGF-comprising Sendai virus is active form.

13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of any patent issuing from the present patent application.

December 5, 2001
Date

Makoto Inoue
Makoto Inoue

APPENDIX A

CURRICULUM VITAE

Makoto Inoue

Current Position: Assistant Manager, Research Scientist, PhD at DNAVEC Research Inc.

Date of birth:	July 6, 1964
Place of birth:	Saga, Japan
Marital status:	Married

Education:

1982 – 1987	B.S., Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
1987 – 1989	M.S., Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
1989 – 1992	D.S., Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
1992 – 1992	Sumitomo Chemical Co. Ltd
1992 – 1999	Sumitomo Pharmaceutical Co. Ltd
1999 – present	DNAVEC Research Inc.

Technical Experience:

Biochemistry
Molecular Biology
Protein Chemistry
Neurochemistry
Virology